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ANTIBACTERIAL ACTIVITY, CHEMICAL COMPOSITION, AND CYTOTOXICITY OF LEAF'S ESSENTIAL OIL FROM BRAZILIAN PEPPER TREE (*SCHINUS TEREBINTHIFOLIUS,* RADDI)

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ABSTRACT

The antibacterial potential of leaf's essential oil (EO) from Brazilian pepper tree (*Schinus terebinthifolius* Raddi) against staphylococcal isolates from dogs with otitis externa was evaluated. The minimum inhibitory concentration of EO ranged from 78.1 to 1,250 µg/mL. The oil was analyzed by GC and GC/MS and cytotoxicity tests were carried out with laboratory animals.

Key words: Essential oil; Brazilian pepper tree; *Schinus terebinthifolius*, otitis externa; *Staphylococcus*.

Essential oils act against microorganisms by causing instability of the plasma membrane leading to rupture of cells (2). This effect is due to array of antimicrobial compounds in oils that are from (but not limited to) terpinoid class as thymol and carvacrol (1). Although antimicrobial activity can be enhanced by a single chemical compound, it usually seems a result of the synergy among many chemical compounds present in oils. For instance, Sonboli *et al*. (2006) described antimicrobial activity in essential oils from *Salvia* species were remarkably dependent of synergy between linalool, 1,8-cineol, alpha-pinene, beta-pinene, beta-caryophyllene and limonene (22). In addition, the range of antimicrobial activity can vary with bacterial species involved. Cristani *et al*. (2007), using a biomembrane model to study essential oil's action mechanism, showed thymol was more toxic against *S. aureus* than carvacrol, p-cymene, and gamma-terpinene, while carvacrol and p-cymene were mainly inhibitory against *E. coli* (5).

The Brazilian pepper tree or Aroeira (*Schinus terebinthifolius* Raddi, Anacardiaceae) is broadly found in Northeast region of Brazil and has been used in popular medicine to treat respiratory infections (12). Previously, Siddiqui *et al*. (21) reported essential oils of this plant carry antibacterial activity against *Escherichia coli, Shigella dysenteriae, Bacillus subtilis* and *Staphylococcus albuns.* Ancient phytochemical studies revealed the presence of triterpene alcohols, ketones, acids, monoterpenes and sesquiterpenes in the bark, leaves and fruits (13). Moreover, a number of studies with leaf´s essential oil of plants collected at different regions of the globe have shown distinct chemotypes by GC/MS analyses, and prevalence of distinct chemical compounds. For example, α-pinene (51,82%) in Indian plants (3), α-phellandrene (24,2%) in Egypt plants (8), limonene (17,7%) and p-cymene (15,7%) in Reunion Island plants (24).

The veterinary use of essential oils depends on clinical

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trials and has been fewer exploited in therapeutics, such as herbal cream containing tea tree oil that was successfully used on symptomatic treatment of canine dermatitis (20). Besides, antimicrobial combinations like chlorhexidine digluconate and tea tree oil or eucalyptus oil and thymol, were useful against biofilm cultures of bacterial isolates of *S. epidermidis* (9). Altogether, in the present study leaf`s essential oil from Brazilian pepper tree was evaluated against clinical isolates of coagulase-positive *Staphylococcus* ssp. from dogs with otitis externa. This essential oil was chemically evaluated by GC and GC/MS analyses and acute toxicity assays were performed using laboratory animals.

Brazilian pepper tree (*Schinus terebinthifolius* Raddi) was collected at North-East Brazil (8º00`52.79" latitude S, 34º57`00.81" longitude W). The voucher species was deposited at the Vasconcelos Sobrinho Herbarium of the university and numbered as 42544. Fresh leaves were submitted to water distillation for 2 h and collected by a Clevenger-type apparatus. The essential oil (EO) was separated from water, dried with Na_2SO_4 and stored in sealed vials at low temperature. GC and GC/MS analysis was carried out as described by Pontes *et al.* (19).

Staphylococci (n = 9) isolates were obtained from the exudates of the ear canals of otopatic dogs being treated at the UFRPE Veterinary Clinic. Identification of isolates used the APIStaph Kit (Biomerieux) and antibiogram susceptibility tests were conducted following CLSI instructions (formally, Committee for Clinical Laboratory Standards) (17). The EO antimicrobial assays were initially carried out by the agar-well diffusion method (18). Staphylococci isolates were cultured in the dishes (10^8 cells/mL - 0.5 of the MacFarland standard) and wells were topped up with 20 µL of essential oil at concentration of 100, 50, 25 and 12.5 mg/mL (w/v) in DMSO (Dimetil Sulfoxide). Such results were expressed as mean ± SD of the growth inhibition zone in millimeters. Differences between inhibition zones (mean ± SD) produced by distinct dosages of the EO were compared by *Student t test* or ANOVA with $P < 0.05$. The minimum inhibitory (MIC) concentration was performed using the broth dilution method in concentrations ranging from 36 to 2 500 µg/mL (10). The MIC was the lowest oil concentration that caused visible inhibition growth; minimum bactericidal concentration (MBC) was the lowest concentration resulting in no growth after the incubation period time of 24h at 37º C. All assays were performed in duplicate.

Male Swiss mice with approximately 35 g in weight were used for acute toxicity tests, following institutional rules stated by the Animal Ethics Committee of the UFRPE. An aliquot of the EO previously dissolved in DMSO was diluted in Tween 80 3% (v/v in Phosphate saline) to produce stock concentrations of 100, 225, 300, 375, 500 and 1000 mg/kg. Then 0.2 mL was administered intraperitoneally to adult Swiss mice (6 animals per group for each dosage). Control animals received only the solvent devoid of essential oil. At the end of experiments the survival animals were sacrificed by anesthesia with Halothane (Halocarbon Laboratories, USA) and submitted to histological examination.

Otitis externa in dogs is caused by multiple factors such as changes in temperature and humidity of the ear canal, accidents with perforating objects, host immunity and infection. The disease therapeutics have been particularly intended to eliminate opportunistic microorganisms that proliferate in the ear canal of healthy as well as otopatic dogs, such as *Staphylococcus intermedius, Streptococcus canis, Escherichia coli, Proteus* sp. and the yeast *Malassezia pachydermatis* (14). In spite of the changes in number and species type of isolates, *Staphylococcus intermedius* have been especially associated with disease and targeted by antibiotic therapy (11). Besides, methicillin-resistant *S. intermedius* (MRSI) and methicillin-resistant *S. schleiferi* (MRSS) were significantly more common in dogs than in cats (16).

In the present study, *S. intermedius* (eight isolates – LMI-S2 to LMI-S9) and *S. schleiferi* (one isolate - LMI-S1) were identified in the ear canal of dogs. The isolate LMI-S8 was resistant to tetracycline, ciprofloxacin, and erythromycin, whereas LMI-S9 was resistant to penicillin, tetracycline, erythromycin and gentamicin. The remaining bacterial isolates were sensitive to all antibiotics. Also, all isolates were sensitive

to oxacilin suggesting the lack of Meticilin-resistant strains. This preliminary assay was useful to typify staphylococcal antibiotic profiles prior to carry out assays with EO from Brazilian pepper tree. The agar-well diffusion technique has shown growth inhibition zones of the EO against staphylococcal isolates until the lower assayed concentration of 12.5 mg/mL (Mean: 7.7 mm ± 0.61). Besides this, a statistical significance was observed among the dosages tested (Table 1). The MIC ranged from 78.1 to 1,250 µg/mL but the oil was not bactericidal to majority of bacterial isolates, except by isolate LMI-S9, which showed MBC corresponding to the higher tested dosage of 2,500 µg/mL.

Table 1. Antibacterial activity of leafs' essential oil from Brazilian pepper tree.

Staphylococci isolates	**Growth inhibition zones in millimeters (mean ± SD)**				**µg/mL****	
	100 mg/mL	**50 mg/mL**	**25 mg/mL**	**12.5 mg/mL**	**MIC**	**MBC**
LMI-S1	17 ± 1.0	9 ± 1.0	8 ± 1.0	8 ± 0.5	156.2	-
LMI-S2	17 ± 1.0	11 ± 1.0	9 ± 1.0	8 ± 0.5	625	-
LMI-S3	11 ± 0.5	10 ± 0.5	8.5 ± 1.0	8.5 ± 0.5	625	-
LMI-S4	11 ± 1.0	11 ± 0.5	9 ± 0.5	8 ± 1.0	1250	-
LMI-S5	16.5 ± 0.5	10 ± 0.5	9 ± 1.0	7.5 ± 0.5	78.1	-
LMI-S6	15.5 ± 0.5	10 ± 0.5	8 ± 0.5	7.5 ± 0.5	1250	-
LMI-S7	10.5 ± 0.5	10 ± 0.5	8 ± 0.5	6.5 ± 0.5	625	-
LMI-S8	17 ± 1.0	11 ± 1.0	10 ± 0.5	8 ± 0.5	312.5	-
LMI-S9	12 ± 0.5	11 ± 0.5	9 ± 0.5	7 ± 0.5	312.5	2,500
Mean ± SD*	14.6 ± 2.88^{a}	10.3 ± 0.7^{b}	8.7 ± 0.66^{c}	7.7 ± 0.61^{d}	-	-

* Different superscript letters mean significant differences at $P < 0.05$.
** MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

The current protocol for control of bacterial infection in otitis externa episodes uses gentamicin administered topically against most of staphylococci isolates and amoxicillin-clavulanic acid for the remaining bacterial species (14). However, an emergence of bacterial isolates carrying mecA gene has been reported for *S. hominis, S. aureus, S. epidermidis, S. haemolyticus* and *S. warneri* in cats and dogs (15). Our data have shown EO from Brazilian pepper tree was active against all coagulase-positive staphylococci obtained from diseased dogs, some resistant to several antibiotics indicated for therapy. Nevertheless, the lack of in vivo pharmacokinetic studies became unfeasible to preview its antimicrobial effects in animal therapeutics. Thus, due to non-bactericidal effect of this oil, it seems to be more appropriated for inclusion in topic preparations or antimicrobial combinations.

In spite of essential oils' bioactive compounds can modify proportionally interfering on antimicrobial ability, GC and GC/MS analyses revealed thirty-three components were identified, representing 95.5% of EO from leaf of the Brazilian pepper tree (Table 2). The main components were p-cymen-7-ol (22.5%); 9-epi-(E)-cariophyllene (10.1%), carvone (7.5%) and verbenone (7.4%). From these p-cymen and caryophyllene were previously reported by its inhibitory effect on microorganisms (4, 6). These findings highlight the importance to elucidate the chemical composition of plant essential oils from distinct geographic regions.

Table 2. Chemical compounds in leaf's essential oil from Brazilian pepper tree.

Compounds	RI[a]	%[b]
o-Cymene	1022	1,7
Limonene	1031	0,2
m-Cymenene	1082	0,7
2,5-Dimethyl styrene	1096	1,0
Perillene	1099	0,5
Myrcenol	1118	0,8
α-Campholenal	1125	0,8
trans-Limonene oxide	1139	3,1
cis-Verbenol	1140	0,9
trans-Verbenol	1144	2,7
m-Cymen – 8 – ol	1180	4,1
p-Cymen-8-ol-	1183	3,2
α-terpineol	1189	0,7
Verbenone	1204	7,4
Carvone	1242	7,5
α-Terpinen -7-al	1282	1,9
p-Cymen-7-ol	1287	22,5
β-dehydro-Eelsholtzione	1298	4,6
α-Cubebene	1351	0,3
neo-dihidro Carveol acetato	1356	0,5
β-Bourbonene	1384	0,1
iso-Longifolene	1402	2,7
cis-Muurola-4(14),5-diene	1460	3,5
9-epi-(E)-caryophyllene	1467	10,1
β-Chamigrene	1475	1,0
γ-Himachalene	1476	0,9
γ-Muurolene	1477	1,8
α-Bulnesene	1507	0,6
Spathulenol	1576	0,6
Caryophyllene oxide	1581	5,2
β-Eudesmol	1649	1,0
α-Cadinol	1653	0,6
Aristolone	1756	2,3

[a] RI, Retention Index.
[b] (%), relative percentage obtained from peak area.

Previously stem bark extracts of the Brazilian pepper tree were reported to produce DNA damage and mutation in bacteria (7). Such toxic effects have been specially evidenced in flavonoid-enriched fractions (23). On the other hand, we were not aware from previous reports describing in vivo cytotoxicity for leaf's essential oil of this plant. In view of toxicological analyses are first step prior clinical trials, we carried out a preliminary evaluation of EO from Brazilian pepper tree. The observation of animal clinical signs after inoculums and histological examination supported the oil was not remarkably toxic. There was no evidence of changes in behavior pattern throughout 24h observation at dosages smaller than 225 mg/Kg. The histological examination showed that coagulative necrosis in kidneys and cell vacuolization plus hyperemia in the liver was limited to dosages higher than 100 mg/Kg (data not shown). Taken together, we conclude the lower toxicity and potent antimicrobial activity of EO from Brazilian pepper tree sustain its potential use for veterinary

practices.

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